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**14. ABSTRACT**
The nuclear receptor estrogen receptor alpha (ERα) is a primary focus of the therapy of breast cancers. ERα-positive breast cancers have traditionally been treated with the anti-estrogen tamoxifen, or with inhibitors of aromatase. ERα-negative breast cancers do not respond to anti-estrogen treatment; instead, current therapeutics, such as Herceptin, have focused on the transmembrane tyrosine kinase receptor ErbB2 (HER2) because it is often overexpressed in ERα-negative tumors and correlates with a poor prognosis. Estrogen-related receptor α (ERRα) has sequence similarity to ERα, but ERRα does not bind estrogens. In some cases ERRα is constitutively active, which is likely determined not only by its interaction with coactivators, but also in part by post-translational modifications occurring via the ErbB2 (HER2) signaling pathways. Thus, ERRα probably plays a key role in the etiology and progression of a subset of breast cancers. We believe that the ERRα-coactivator interaction is a promising target for new chemotherapeutic drug development. We have generated and characterized a set of murine monoclonal antibodies for use in studying the biology of ERRα. We are now using some of those antibodies in gentle immunoaffinity chromatography to determine ERRα-associated proteins. Using *in vitro* assays, we will study the binding properties of ERRα with known coactivators like transcriptional intermediary factor 2 (TIF2), as well as any ERRα-associated proteins we may find during immunopurification. In the future, using a luminescence resonance energy transfer-based high-throughput screen previously developed in our laboratory, we hope to identify small molecules that interfere with the binding of ERRα with these coactivators.

**15. SUBJECT TERMS**
Estrogen-related receptor alpha (ERRα), gentle immunoaffinity chromatography, protein-protein interactions, Luminescence Resonance Energy Transfer (LRET)

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**TABLE OF CONTENTS**

## INTRODUCTION

The nuclear receptor estrogen receptor alpha (ERα) is a primary focus of the therapy of breast cancers. Its transcriptional activity is modulated by its binding to the steroid hormone estrogen, which allows ERα to bind estrogen response elements (EREs) in gene promoter regions. Many of its target genes, such as progesterone receptor (PgR), are involved in cell proliferation, and deregulation of ERα can lead to uncontrolled cellular growth and the promotion of breast cancer. ERα-positive breast cancers have a better prognosis and have traditionally been treated with the anti-estrogen tamoxifen, or with inhibitors of aromatase, an enzyme involved in estrogen synthesis. ERα-negative breast cancers, which occur in 25% of patients, do not respond to anti-estrogen treatment. Current therapeutics for ERα-negative cancers, such as Herceptin, have focused on ErbB2 (HER2) as a target because this transmembrane tyrosine kinase receptor is often overexpressed in ERα-negative tumors and correlates with a malignant phenotype and poor prognosis. Estrogen-related receptor α (ERRα) is a protein with sequence similarity to estrogen receptor α (ERα), but ERRα does not bind estrogens. However, ERRα can still bind to estrogen-response elements (EREs) in the promoter region of certain genes, and in some cases function as a constitutive activator, with its activity likely determined not only by its interaction with coactivator proteins, but also in part by post-translational modifications occurring via the ErbB2 (HER2) signaling pathways. Thus, ERRα probably also plays an important role in the etiology and progression of a subset of breast cancers.

The long-term goal of this project is to find a new treatment for human breast cancers, particularly aggressive ones that have constitutively active ERRα and are ErbB2 (HER2) overexpressing, ERα-negative, or tamoxifen-resistant. We believe that the ERRα-coactivator interaction is a promising target for new chemotherapeutic drug development. Therefore, using gentle immunoaffinity chromatography, we will determine ERRα-associated proteins; using *in vitro* assays, we will study the binding properties of ERRα with coactivators; and using a luminescence resonance energy transfer (LRET)-based high-throughput screen previously developed in our laboratory, we will identify small molecules that interfere with the binding of ERRα with these coactivators.

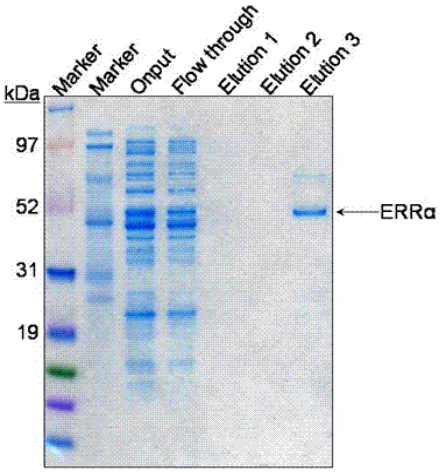
## BODY (RESEARCH ACCOMPLISHMENTS)

### Task 1: Determine ERRα-associated proteins.

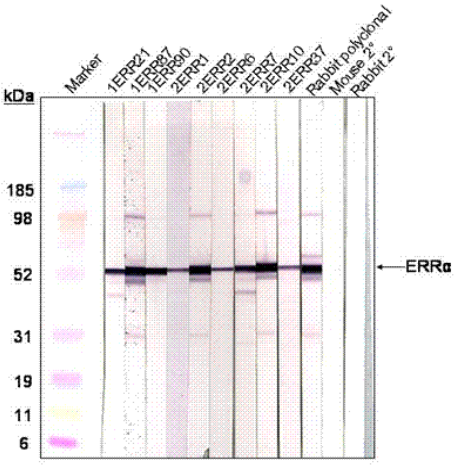
*Previously reported findings:* We reported that we were in the process of producing murine monoclonal antibodies (mAbs) to estrogen-related receptor alpha (ERRα).

*Generation of monoclonal antibodies:*

To make the immunogen, human cDNA of ERRα1 was inserted into the overexpression plasmid pET28b(+) (Novagen). The human ERRα1 gene with a C-terminal hexahistidine-tag was encoded and expressed from a T7 promoter in *Escherichia coli* and purified by a nickel-nitrilotriacetic acid (Ni-NTA) affinity column (Figure 1). Using standard hybridoma techniques, a panel of murine mAbs was produced to the hexahistidine-tagged ERRα. Nine of those mAbs were tested for specificity in a Western blot (Figure 2).




←**Figure 1.** Stained SDS-PAGE of Ni-NTA affinity column purification of C-terminally hexahistidine-tagged ERRα expressed in *E. coli* BL21(DE3) cells.




→**Figure 2.** Composite Western blot of anti-ERRα mAbs from hybridoma supernatants binding to ERRα overexpressed in MCF-7 cells. Rabbit polyclonal antibody, called YC2, was developed previously in the Mertz laboratory.

*Polyol-responsiveness of the monoclonal antibodies:*

The nine mAbs were tested to see if any were polyol-responsive, meaning the mAb binds its antigen tightly but releases it under gentle, non-denaturing conditions in the presence of an aqueous buffer at neutral pH supplemented with a low-molecular-weight polyhydroxylated compound (polyol) and a non-chaotropic salt. We have shown that the advantages of using polyol-responsive monoclonal antibodies (PR-mAbs) during immunoaffinity chromatography are two-fold: enzymes eluted under these gentle conditions retain their biological activity; and protein complexes maintain their structural integrity, allowing subsequent purification of any resulting multi-protein complex by size exclusion or ion exchange chromatography (for review, see Burgess and Thompson, 2002). In our experience, about 5-10% of all mAbs are polyol-responsive; however, we were pleasantly surprised to find that five of the nine tested anti-ERRα mAbs were polyol-responsive (Table 1). In a modified ELISA assay, these five antibodies bound tightly to 96-well polystyrene plates that had been coated with His-tagged ERRα and blocked with 1% dry milk in phosphate-buffered saline, but the mAbs were released from the antigen in the presence of a buffer (50 mM Tris-HCl, pH 7.9, 0.1 mM EDTA) containing the following four tested salt/polyol combinations: 0.75 M ammonium sulfate and 40% propylene

glycol; 0.75 M ammonium sulfate and 40% ethylene glycol; 0.75 M sodium chloride and 40% propylene glycol; 0.75 M sodium chloride and 40% ethylene glycol.

*Most recent findings:* We have made significant progress and are very near the completion of extensively characterizing this set of nine mAbs. We felt it was critical to know some of the properties before proceeding to use these antibodies in gentle immunoaffinity chromatography.

*Epitope mapping of the monoclonal antibodies:*

Using Western blot, we have been able to roughly determine the epitope of five of the nine mAbs. Figure 3 shows examples of the mapping of two mAbs. Equal amounts of MCF-7 whole cell extract containing various versions of overexpressed ERRα (Figure 3A) was separated by SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was blocked with 5% dry milk in phosphate-buffered saline. In Figure 3B, left, mAb 2ERR10, purified from ascites fluid, was used as the primary antibody in the Western blot at a concentration of approximately 1 µg/ml. In Figure 3B, right, mAb 2ERR7 hybridoma supernatant, at a dilution of 1:3, was used as the primary antibody in the Western blot. In both cases, a horseradish peroxidase-conjugated goat-anti-mouse IgG secondary antibody was used at a concentration of 1 µg/ml. ECL reagents were used for detection. mAb 2ERR10 reacts with full-length ERRα, 1-376, 1-198, and 1-173, but does not interact with ERRα 77-423. This pattern suggests that the epitope of mAb 2ERR10 is in the N-terminus of ERRα, specifically between amino acids 1-76. In a similar analysis, mAb 2ERR7 interacts with full-length ERRα, 1-376, and 77-423, but it does not interact with ERRα 1-198 or 1-173, suggesting that its epitope is between amino acids 199-376. All other antibodies were subjected to the same analysis as these two antibodies, and similar patterning was seen (Western blots not shown).

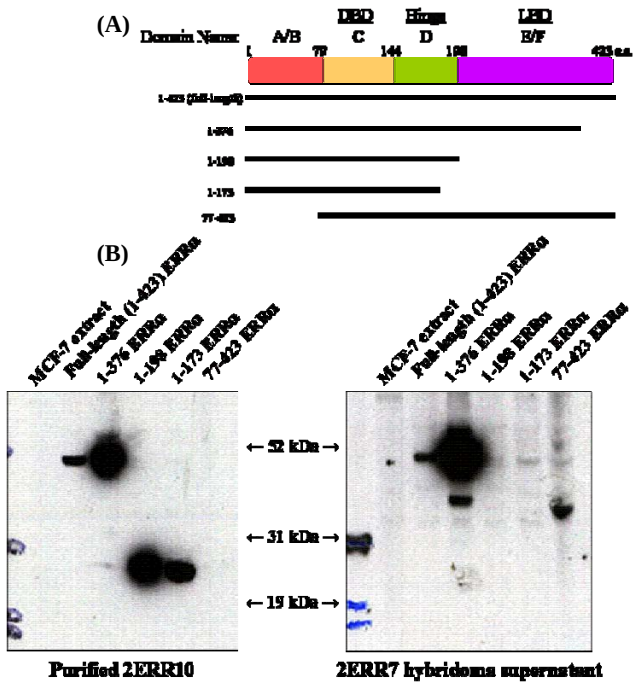



**Figure 3. (A)** ERRα constructs transiently transfected into MCF-7 cells. Amino acids 1-423 is full length protein. ERRα 1-376 has the far C-terminal activation function 2 (AF2) region, a common protein-protein interaction site with the p160 coactivators, removed. ERRα 1-198 has the entire ligand binding domain (LBD) removed, whereas ERRα 1-173 has in addition part of the hinge region removed. Lastly, ERRα 77-423 has the A/B domain removed, which includes the AF1 region, another common site of protein-protein interactions. All constructs contain the DNA binding domain (DBD). **(B)** Western blot using either purified mAb (e.g. 2ERR10) or hybridoma supernatants (e.g. 2ERR7) and binding to versions of ERRα overexpressed in MCF-7 whole cell extracts. Equal amount of total whole cell extract protein was loaded in each lane.

*Immunoprecipitation using the monoclonal antibodies:*

We next wanted to see if the mAbs could work in immunoprecipitation experiments, which we thought would represent "mini" immunoaffinity columns and therefore give us some insight as to which mAb would work in our larger gentle immunoaffinity chromatography

procedure. Figure 4 shows one example of the immunoprecipitation experiment performed. Vero cells, which are African green monkey kidney cells and seem to be ERRα-negative, were transiently transfected with full-length ERRα. Cells were collected 48-hours post-transfection, and whole cell extract was produced. Anti-ERRα mAbs (e.g. 2ERR10) were attached to anti-mouse IgG-coated magnetic beads (Novagen). This bead/antibody complex was added to Vero whole cell extract and allowed to incubate at room temperature for one hour. The beads were extensively washed with phosphate-buffered saline and then boiled in SDS-loading buffer. The samples were run on SDS-PAGE and proteins were transferred to nitrocellulose. The membrane was blocked with 5% dry milk in phosphate-buffered saline. An anti-ERRα rabbit polyclonal antibody, called YC2 and developed in the Mertz laboratory, was used at a concentration of approximately 1μg/ml as the primary antibody in the Western blot. A horseradish peroxidase-conjugated goat anti-rabbit IgG, at 0.2 μg/ml concentration, was used as the secondary antibody, and ECL reagents were used in the detection. As seen in Figure 4, mAb 2ERR10 specifically precipitates ERRα from the Vero whole cell extract. mAbs 1ERR21 and 1ERR87 were tested in a similar way and also found to specifically pull-down ERRα. Unfortunately, 2ERR6 does not seem to be able to pull-down ERRα from a whole cell extract, although we are in the process of repeating this experiment with this antibody as well as finishing these immunoprecipitation experiments with the rest of the mAbs.

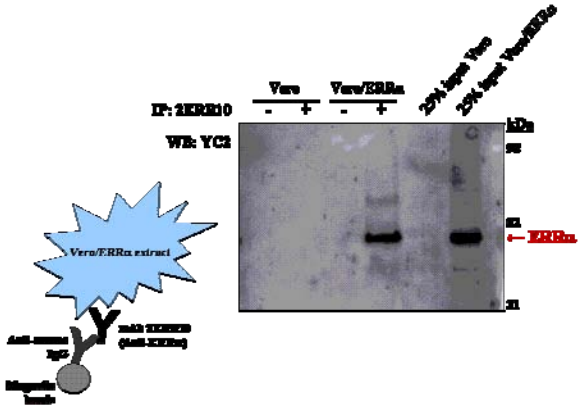



**Figure 4.** Immunoprecipitation using mAb 2ERR10 of either Vero whole cell extract (Vero) or Vero whole cell extract containing overexpressed full-length ERRα (Vero/ ERRα). Western blot using anti-ERRα rabbit polyclonal antibody YC2.

*Size-exclusion chromatography and analysis using the monoclonal antibodies:*

In preparation for producing immunoaffinity columns, we are generating larger quantities of the polyol-responsive mAbs by either growing the hybridoma cells in cell culture flasks or by producing ascites fluid in mice, and isolating the antibodies using standard purification techniques. Already, we have purified tens of milligram quantities of 1ERR21, 2ERR1, 2ERR6, 2ERR10, and 2ERR37. Immunoaffinity chromatography resins will be produced by using cyanogen-bromide activated Sepharose 4B (Sigma-Aldrich) to covalently attach the mAbs to the resin using methods described in Thompson and Burgess (2001). We are also isolating HeLa nuclear extract. We have received a generous gift of nuclear extract from 12 L of HeLa cell culture from Dr. Al Courey at UCLA. We also plan on purchasing more HeLa cell nuclei from the National Cell Culture Center. We have run preliminary experiments for determining the complexes in which ERRα is participating in HeLa cells. HeLa nuclear extract was run over a size exclusion column (Sephacryl S300), and fractions were collected. Samples of the fractions were dotted onto nitrocellulose, which was then blocked with 1% dry milk in phosphate-buffered saline. Fluorescently-labeled

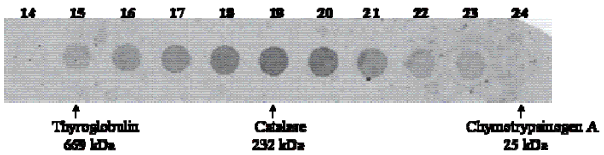



**Figure 5.** Dot blot (Western blot) of fractions # 14-24 collected from size exclusion chromatography. Fluorescently labeled mAb 2ERR10, at 1 μg/ml final concentration, was used as the immunodetection agent. Thyroglobulin, catalase, and chymotrypsinogen A are known molecular weight markers. A fluorimager was used for detection.

mAb 2ERR10 was used as the immunodetection reagent in the Western blot shown in Figure 5. Based on the elution of known molecular weight markers (thyroglobulin, catalase, and chymotrypsinogen A) from the column, it appears that ERRα is predominantly in a complex of approximately 250 kDa in HeLa nuclear extract.

*Supershift assays using the monoclonal antibodies:*

We have recently determined which of the mAbs can supershift a protein-DNA complex in electrophoretic mobility shift assays (EMSAs) (Figure 6). MCF-7 whole cell extract overexpressing full-length ERRα was incubated with or without purified mAb or mAb-enriched ascites fluid. The monoclonal antibody NT73, specific to *Escherichia coli* RNA polymerase subunit β', was used as a negative control; the anti-ERRα polyclonal antibody YC2 was used as a positive control. The protein or protein-Ab complexes were then incubated with a radiolabeled DNA probe containing the consensus steroidogenic factor 1 response element (SFRE) sequence. Samples were run on native gels, exposed to a phosphorimager screen, and visualized on a Storm Imager. As shown in Figure 6, 1ERR87, 1ERR90, 2ERR1, and 2ERR10 can supershift the ERRα-DNA complex, but 1ERR21, 2ERR6, 2ERR7, and 2ERR37 cannot shift the complex. We are encouraged by these results, and we are now determining if these mAbs can supershift an ERRα-DNA complex in different whole cell extracts, such as BT-474 and HeLa cells, where ERRα is known to be a constitutive activator of transcription.

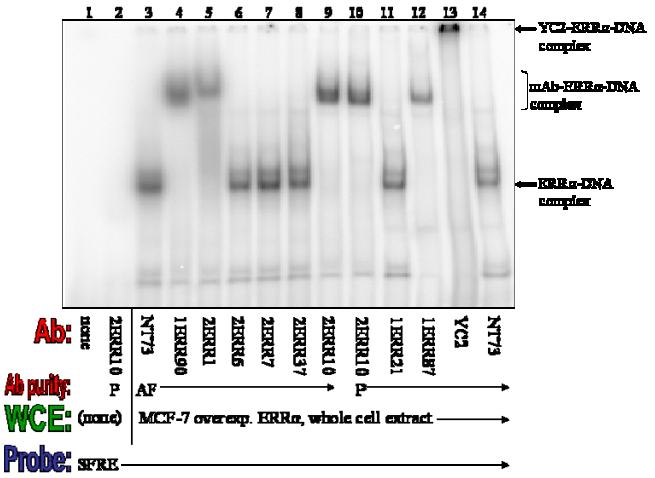



**Figure 6.** Supershift assay using anti-ERRα mAbs (Ab), either purified (P) or enriched ascites fluid (AF); MCF-7 whole cell extract (WCE) overexpressing full-length ERRα; and a radiolabeled DNA probe (Probe) containing the steroidogenic factor response element (SFRE). Equal amounts of mAb, whole cell extract, and probe were added to each lane.

*Immunohistochemistry and chromatin immunoprecipitation:*

Lastly, we started a collaboration in November 2005 with Dr. Anastasia Kralli at The Scripps Research Institute to test some of our mAbs in immunohistochemistry (IHC) and chromatin immunoprecipitation (ChIP) experiments. We look forward to seeing those results.

**Table 1.** Summary of anti-ERRα monoclonal antibody characterization

| mAb | 1ERR21 | 1ERR87 | 1ERR90 | 2ERR1 | 2ERR2 | 2ERR6 | 2ERR7 | 2ERR10 | 2ERR37 |
|---|---|---|---|---|---|---|---|---|---|
| **Purified?** | Yes | Yes | No | Yes | No | Yes | No | Yes | Yes |
| **Isotype** | $IgG_1$ | $IgG_1$ | $IgG_1$ | $IgG_{2a}$ | $IgG_{2a}$ | $IgG_{2b}$ | $IgG_{2b}$ | $IgG_1$ | $IgG_1$ |
| **WB** | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| **PR-mAb** | Yes | No | No | Yes | No | Yes | No | Yes | Yes |
| **Epitope** | 199-376 | 1-76 | 1-76 | nd | nd | nd | 199-376 | 1-76 | nd |
| **IP** | Yes | Yes | nd | nd | nd | No | nd | Yes | nd |
| **Supershift** | No | Yes | Yes | Yes | nd | No | No | Yes | No |
| **IHC *** | | | | | | | | | |
| **ChIP *** | | | | | | | | | |

**ERRα:** A 423-amino acid nuclear receptor protein related to estrogen receptor.
**Purified:** purified from ascites fluid using methods previously described in Thompson and Burgess (2001).
**Isotype:** Type of immunoglobulin heavy chain; antibody subtype.
**WB:** Western blot; suitable for use in immunoblotting to detect protein expression.
**PR-mAb:** Polyol-responsive monoclonal antibody; able to bind antigen with high affinity but release under non-denaturing conditions.
**Epitope:** Location of mAb binding site on full-length ERRα (amino acids 1-423); region from amino acid # to amino acid #.
**IP:** Immunoprecipitation; suitable for use in immunoprecipitation to isolate ERRα and any associated proteins from cell extracts.
**Supershift:** Able to detect ERRα in its DNA-bound form in a gel shift assay.
**IHC:** Immunohistochemistry; useful in identifying cells in a tissue sample that contain ERRα.
**ChIP:** chromatin immunoprecipitation; able to immunoprecipitate ERRα in its chromatin-bound form from cell extracts.
**nd:** not determined.
***** Will be determined in collaboration with Dr. Anastasia Kralli, of The Scripps Research Institute

## Task 2: Study the binding properties of ERRα and its coactivators.

Because we have not yet isolated a transcriptional complex containing ERRα and its associated proteins (Task 1), we have not been able to start Task 2. We are, however, beginning to study the binding properties of ERRα and a member of the p160 family of transcriptional coactivators, TIF2. Please see our research accomplishments in Task 3 for more details.

We have added to our knowledge of ERRα's transcriptional activity in another breast cancer cell type: SK-BR-3. The Mertz laboratory has previously published that in MCF-7 cells full-length ERRα is a repressor of ERE-dependent transcription, and in BT-474 cells ERRα is a constitutive activator of ERE-dependent transcription (Kraus *et al.*, 2002). We have found that in SK-BR-3 cells, full-length ERRα is a repressor of ERE-dependent transcription; however, an N-terminal truncation of ERRα, in the presence of the coactivator GRIP1 (TIF2, SRC-2), is an activator of ERE-dependent transcription. Please see our research accomplishments in Task 3 for more details on this N-terminal truncation of ERRα.

## Task 3: Develop a high-throughput screen for molecules that interfere with the binding of ERRα and a coactivator.

It has been well-established in the Mertz laboratory that in MCF-7 cells ERRα functions as a repressor of transcription, and in BT-474 cells ERRα functions as a constitutive activator of transcription. However, very new and exciting unpublished transcriptional data from the Mertz laboratory suggests a N-terminal truncated version of ERRα converts ERRα from a repressor to

an activator in MCF-7 cells on an ERE-luciferase reporter gene. This suggests that there is a repressor motif in the N-terminus of ERRα. Unfortunately, we have not yet been able to co-immunoprecipitate the N- and C-terminus of ERRα using our monoclonal antibodies, which suggests that (1) we may have not yet optimized the proper binding conditions, or (2) there may be a bridging protein required for the N- and C-terminus of ERRα to interact.

The Mertz laboratory has additionally found that GRIP1, but not the other p160 family of transcriptional coactivators SRC-1 or SRC-3, is a major coactivator of full-length ERRα in BT-474 cells, where ERRα is known to be constitutively active. GRIP1, but again not SRC-1 or SRC-3, is also the major coactivator of the N-terminal truncated ERRα in MCF-7 cells. In both BT-474 and MCF-7 cells, GRIP1 further potentiates transcription of ERRα on an ERE-luciferase reporter gene, but most interestingly GRIP1 does *not* increase ERRα's transcriptional activity on an SFRE-luciferase reporter gene. This observation suggests that ERRα specifically interacts with GRIP1 only when it is bound to an ERE; in other words, the ERRα/GRIP1 interaction is ERE-dependent. Other researchers have been able to show a protein-protein interaction of GRIP1 and ERRα in GST-pulldown experiments and have supported our observation that ERRα/GRIP1 interactions activate transcription synergistically in transient transfection assays (Xie *et al.*, 1999; Lu *et al.*, 2001; Zhang and Teng, 2001).

We are encouraged by these findings and would like to pursue this protein-protein interaction as a target for new chemotherapeutic development. We are currently cloning the human homolog of murine GRIP1, transcriptional intermediary factor 2 (TIF2), into bacterial and mammalian expression vectors. TIF2 and GRIP1 share 90% sequence similarity and are often substituted for one another in experiments; nevertheless, we would like to work with human proteins if we are to develop new anti-breast cancer therapeutics. We will characterize the transcriptional activity of TIF2 and ERRα, which we believe will be very similar to GRIP1 and ERRα. We hope to soon begin working on Tasks 2 and 3 with the ERRα/TIF2 protein-protein interaction.

We hope to start a cell-based high-throughput screen with the Small Molecule Screening Facility at the UW-Comprehensive Cancer Center on campus within the next 4-6 months. We will co-transfect TIF2, ERRα (or truncated ERRα), and an ERE-luciferase reporter plasmid into BT-474 cells (or MCF-7 cells). We will screen for small molecules that interfere with ERRα/TIF2 interaction and thus inhibit transcription of the reporter plasmid and decrease overall luciferase activity.

## KEY RESEARCH ACCOMPLISHMENTS

- We have prepared a set of nine murine monoclonal antibodies against ERRα.
- We have characterized these mAbs extensively.
- We have started to study the interaction of ERRα with TIF2, a member of the p160 family of transcriptional coactivator proteins.

## REPORTABLE OUTCOMES

**Jennifer A. Lamberski**

Abstracts:

- Great Lakes Nuclear Receptor Conference; Madison, WI; October 15, 2005
- The Keystone Symposia, Nuclear Receptors: Orphan Brothers; Banff, Alberta, Canada; March 18-23, 2006

Additional funding:

- Vilas Travel Award from the University of Wisconsin-Madison ($600 award for travel to the Keystone Symposia)

Licenses:

- Disclosed the anti-ERRα mAbs to the University of Wisconsin-Madison patenting and licensing group, WARF

Poster Sessions:

- Great Lakes Nuclear Receptor Conference; Madison, WI; October 15, 2005
- The Keystone Symposia, Nuclear Receptors: Orphan Brothers; Banff, Alberta, Canada; March 18-23, 2006

Presentations:

- Cancer Biology Student/Postdoc Seminar Series; February 1 and December 20, 2005
- Molecular and Cellular Pharmacology Student Seminar Series; April 11 and December 5, 2005
- Nuclear Receptor Group meeting, September 30, 2005

## CONCLUSIONS

We have made significant progress this year in generating and characterizing nine murine monoclonal antibodies against ERRα. We have determined their isotypes, epitopes, and polyol-responsiveness, and have tested the antibodies in a variety of assays including Western blot, immunoprecipitation, supershift, immunohistochemistry, and chromatin immunoprecipitation. Importantly, we have identified the antibodies most likely to work in gentle immunoaffinity chromatography, which will help us complete Task 1, finding ERRα associated proteins.

The Mertz laboratory has recently produced data suggesting that ERRα/GRIP1 interactions are important in driving ERE-dependent transcription in breast cancer cells. We look forward to pursuing this idea further by cloning the human homolog of GRIP1 into bacterial and mammalian expression vectors and studying its binding interactions with ERRα. This will help us continue our research in Tasks 2 and 3, studying the binding interactions of ERRα and a coactivator protein and developing a high-throughput screen for small molecules that interfere with this interaction.

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